Saskatchewan

A D F

Agriculture Development Fund

FINAL REPORT

PREGNANCY RATES WITH CRYOPRESERVED EQUINE SEMEN

19990131

Funded by
The Agriculture Development Fund

February 2001
Prepared by: University of Saskatchewan

SAF
Saskatchewan Agriculture & Food

Adding value to agriculture...through research and development

ADF Report Project Number 19990131

Pregnancy Rates with Cryopreserved Equine Semen

Dr. C. Card, Dept Large Animal Clinical Sciences, Western College of Veterinary Medicine, 52 Campus Drive, Saskatoon, SK S7N 5B4 306-966-7102, 306-966-7159 fax

Long Term Objective: to develop guidelines for the use of cryopreserved equine semen.

To work towards this goal the following specific aims were investigated:

Specific Aims:

1. To determine differences in per cycle pregnancy rates using single insemination dosages based on post thaw: sperm cell motility, morphology, acrosome status and longevity

2. To formulate rationale guidelines and objective quality standards for cryopreserved equine semen

Background Description of the Problem:

The development of new technology such as the ability to chill or cryopreserve semen, has resulted in a large increase in the number of mares bred using artificial insemination (AI). The number of breed associations approving AI with cooled transported or cryopreserved semen continues to expand. Cryopreservation of equine semen offers many potential advantages over natural breeding, or artificial insemination of fresh or chilled transported semen. The major benefits include increased accessibility, security, and efficiency. Cryopreserved semen is convenient from the mare owner's perspective because it is: stable in storage, constantly available, easy to transport from anywhere in the world, and has fewer regulatory barriers restricting its movement compared to animals. Therefore with frozen semen mare owners may select stallions from distant countries, and are no longer limited to domestic stallions or stallions from neighboring countries. From a stallion owner's perspective, cryopreserved semen is efficient because it can be collected and stored from stallions in high demand during the non-breeding season, or during periods when they are not in competition. Cryopreserved semen is also efficient because more than one breeding dose may be obtained from each ejaculate. A popular stallion's book may be expanded by having more semen available for the breeding season by collecting and freezing semen when there are no mares to breed. Compared to the organizational tasks associated with collecting and shipping cooled transported semen to mares at short notice, cryopreserved semen, because of its stability, is much easier to transport. It is typically sent before the anticipated heat. Cryopreserved semen offers

SEP 15 2000
REGINA, SASK.
S4S 0B1

security for owners of genetically superior stallions. This is because frozen semen may be stored indefinitely. A stallion's genetic material is therefore already preserved in case of illness or accidental death.

There are problems associated with the use of cryopreserved equine semen including: intra- and inter- stallion variability in the post thaw viability of sperm cells, lack of quality control standards, intensive mare management, expense, need for expertise, and generally disappointing per cycle conception results. Per cycle conception rates with frozen equine semen are highly variable and range from 0 - 50%, with most authors reporting rates around 20%. The quality of cryopreserved semen is evaluated in most species by assessing post thaw sperm cell concentration, motility characteristics, morphology, acrosome status, sperm cell longevity, and penultimately is quantified through pregnancy rates. Most insemination doses of frozen equine sperm cells are based entirely on the number of motile sperm cells immediately post thaw. This is an easy characteristic to quantify by microscopic examination of a drop of sperm. However there is wide variability in other characteristics of the cryopreserved sperm cells such as the percentage of morphologically normal, acrosome intact sperm cells present in semen. The percentage of normal sperm cells may vary in a stallion by season, year and ejaculate. The majority of stallions have about 50% morphologically normal sperm cells, but there is a range of 0 - 95%. The lower percentage of normal sperm cells in stallions compared to other species, such as bulls, is believed to relate in part to the focus of the equine industry on the selection of stallions based on performance, color, pedigree, or conformation traits rather than or along with selection for fertility. The vast majority of sperm cell morphologic defects are a result of testicular and epididymal processes, and are not an artifact of the freezing process. Many of these abnormal sperm cells have extreme aberrations, such that they are incapable of forward motion or fertilization. One morphologic defect that is usually associated with processing is acrosome damage. The acrosome is a part of the membrane that covers the top half of the sperm cell head. This structure is sensitive to freezing damage. Therefore during an evaluation of frozen semen the percentage of damaged versus intact acrosomes is calculated as an indicator of the damage associated with processing. Damage to the acrosome also influences the lifespan of the sperm cell. Membrane changes, called capacitation, are necessary before a sperm cell can fertilize an ovum. Cryopreservation may cause capacitation like changes in the membranes of sperm cells that may shorten their lifespan. The longevity of frozen thawed equine sperm at body temperature is used as an indicator of the health of the membranes following cryopreservation. Keeping a sperm cell at body temperature constitutes a stress, and a hardy sperm cell should survive 2 hours at this temperature. Testing the longevity of sperm at this temperature is regarded as a means of providing valuable information on its vigour. There are few reports of breeding trials using cryopreserved equine semen. Comparisons using this information are difficult to interpret because the insemination dosages were not well characterized in terms of sperm cell concentration, motility, morphology, acrosome status, longevity and in the number and timing of inseminations. It is common in these breeding trials to have multiple inseminations per mare in one heat. In practice, the goal is to breed each mare with one appropriately timed insemination dose near ovulation. Mares have the ability to store sperm cells in their uterine tubes, hence in order to determine an appropriate insemination dose a single breeding should be used. The relative importance of motility, ratio of normal to morphologically defective sperm, longevity, and acrosome status in equine semen has been debated. This is likely related to the very large

numbers of sperm cells that are inseminated in mares (100 - 500 million motile normal cells per insemination dose). Only a fraction of this number of normal motile cells are required for good pregnancy rates. If a certain minimum critical threshold of normal motile cells is present in an insemination dose, the normal cells will compensate to some degree for the abnormal cells, with the end result being fairly acceptable conception rates. This concept is termed compensability. Studies in horses have shown that normal sperm cells selectively enter the uterine tube. Fertile stallions have more sperm enter the uterine tube than subfertile stallions, suggesting again the most important characteristic is the number of normal motile cells.

The equine industry is an important industry in Canada because it employs more people than the airline industry. Reproductive inefficiency plagues this industry and costs breeders millions of dollars every year. Valuable genetic material is currently under utilized because of the inability to make semen cryopreservation economically viable, and because of mare and stallion subfertility. In some breeds such as Thoroughbreds, The Jockey Club reports that only 55% of all bred mares foal.

Experimental Design

General Hypothesis: Careful characterization of post thaw viability of equine sperm cells is an important factor in obtaining good pregnancy rates with frozen semen

Broad objective: To optimize conception rates using cryopreserved semen

To compare conception rates using insemination doses calculated based on post thaw: sperm cell motility, morphology, acrosome status, and longevity

To use this information to make rational guidelines and objective quality standards for the use of cryopreserved equine semen

Experiment 1: The effect of insemination dose on pregnancy rates using frozen semen

Method:

Young mares (n = 40) with no history of reproductive problems were used for a total of 55 cycles. In 5 cycles the mares were not bred because they ovulated very early post treatment with human chorionic gonadotropin (hCG). Data from the synchronization aspect of the trial were available for 55 cycles, therefore data from bred mares were available from 50 cycles. A few mares were bred over 2 cycles. Mares were synchronized using 2 shots of Lutalyse 5 mg SQ (UpJohn) 14 days apart. Mares were teased daily, palpated, and evaluated using ultrasound 2x day during estrus. They were treated using hCG (2000 IU APL IM, Wyeth Ayerst) and inseminated once 36 hours later. Mares werethen evaluated at about 12 hours post insemination, and then if ovulation was not detected they were examined once daily until ovulation was detected. Mares were also monitored for post breeding fluid accumulation. In cycles where the ovulation occurred in the 24 hour period after hCG administration, the mares were short cycled with prostaglandin and

then bred.. Pregnancy status was determined at 14 days post ovulation using ultrasonography. The examination before breeding, and the 12 hour post breeding examination were recorded on S-VHS videotape. These images of the endometrial tissues were then analyzed later to look for subtle inflammation, using a computer assisted image analysis system and software (Synergyne, Dr. R. Pierson).

Sperm cells from a stallion with good post thaw characteristics were used based on the number of progressively motile morphologically normal (PMMN) spermatozoa at 3 dosages to comprise 3 groups:Group 1 - 250 million PMMN n = 19, group 2 -100 million PMMN n = 19, and group 3 - 25 million PMMN cells n =12. Frozen semen was thawed at 37 C in a water bath. At the time of hCG administration each group of three mares was randomized between the insemination doses by pulling numbers from an envelope. All mares from one randomization were inseminated with semen from the same ejaculate and the order of the ejaculate use was randomized throughout the trial period. The inseminator was blinded to the dose of the ejaculate used. To standardize the volume the thawed semen was mixed with Kenney's extender to achieve a final volume of 20 mls.

Results:

Semen Cryopreservation

Twelve stallions were evaluated as potential candidates for the cryopreserved semen. One stallion with an excellent per cycle pregnancy rate using natural service and artificial insemination (AI) was chosen for use. From the selected stallion thirteen ejaculates were cryopreserved and 10 of these met the criteria for post thaw use. The mean percentages of motile sperm were 34% and 27% and the mean percentage of normal acrosomes was 83% and 77% at 10 minutes and 2 hours post thaw.

Mare Synchronization of Estrus using 2 injections of Prostaglandin 14 days apart and Ovulation Induction using Human Chorionic Gonadotropin (hCG)

Sixty-eight percent of the mares showed estrus within 3 days of the second prostaglandin injection, and 77% showed estrus within 6 days of the second prostaglandin treatment. The use of hCG for synchronization of ovulation was successful (Table 1). In the majority of cycles (40/55) the mares ovulated within (before or after) 12 hours of artificial insemination. Mares were inseminated at 36 hours after hCG.

Table 1: Interval from treatment with hCG to ovulation in 55 cycles from 40 mares used in a breeding trial with cryopreserved equine semen.

Interval from hCG treatment to ovulation	cycles
<24 hrs*	5*
24 - 36 hrs	8
36 - 48 hrs	32
48 - 72 hrs	6
> 72 hrs	4

*in these 5 cycles insemination was not performed

Table 2 shows the pregnancy rate in 50 cycles from 40 mares using cryopreserved equine semen at three different dosages of normal and progressively motile sperm cells

Table 2: Results of the breeding trial showing numbers of progressively motile morphologically normal (PMMN) spermatozoa and per cycle pregnancy rate

Group 1	250 million PMMN	3/19	15%
Group 2	100 million PMMN	2/19	10%
Group 3	25 million PMMN	1/12	8%

Pregnancy rates were not significantly different between the 3 insemination doses when compared using Chi square analysis. There were more pregnancies in mares inseminated with the larger number of PMMN sperm cells. Five of the 6 mares that became pregnant ovulated close to insemination with the majority at insemination or within 12 hours of breeding. One ovulated 12 to 36 hours after insemination.

Summary:

Following the process of cryopreservation the equine semen was subjected to in vitro testing similar to that which occurs with bovine semen. The guidelines for the evaluation of bovine cryopreserved semen were developed by Dr. Albert Barth of the Spermatology Laboratory of the WCVM. It was not known whether equine semen could be held to the same standard as bovine semen, or whether it would survive the stress of the testing process. The evaluation of frozen semen in the bull involves thawing the semen, and holding it at 37 C (body temperature for 2 hours) in a water bath. The motility of the semen is then evaluated at 10 minutes and 2 hours. The status of the sperm cell membrane called the acrosome is also evaluated at these time points. The acrosome is a structure that is susceptible to freezing damage. A large decrease in the number of normal acrosomes during incubation in the water bath indicates that the semen was damaged in the cryopreservation process. We concluded that equine semen is capable of meeting the same quality control standards as that of bovine semen. However as the general fertility of stallions is lower than that of bulls, a better job of cryopreservation had to be performed with poorer quality semen to achieve acceptable post thaw spermatozoal characteristics.

Synchronization was not a limiting factor in the breeding trial as 68% of the mares showed estrus within 3 days of the second prostaglandin injection, and 77% showed estrus within 6 days of the second prostaglandin treatment. Response rates to hCG were high as 74% of the mares ovulated between 24 and 48 hours after hCG treatment. Pregnancy rates of 15, 10, and 8% were obtained for the three insemination dosages. Differences in pregnancy rates were not significant between the 3 insemination doses when compared using Chi square analysis. There were more pregnancies in mares inseminated with higher dosages of sperm cells. Five of the 6 mares that became pregnant ovulated at insemination or within 12 hours of breeding. One ovulated 12 to 36 hours after insemination. The highest insemination dose(250 million PMMN) is similar to what many practitioners are using as an insemination dose. Overall per cycle pregnancy rates by practitioners are reported as 20%, hence the 15% per cycle pregnancy rate we achieved

in group 1 was close to that value. It is therefore suggested by the results of this study that higher amounts of PMMN spermatozoa need to be inseminated to increase pregnancy rates.

Take home message:

Most studies on cryopreserved semen use daily insemination until ovulation with an undefined number of morphologically normal spermatozoa. The use of multiple inseminations makes it impossible to determine the minimum number of normal motile spermatozoa to achieve good pregnancy rates.

This trial was the first study to show that equine semen could be evaluated in vitro using the same standards as those applied to bull semen. This is also the first trial to use a large number of mares with a defined amount of normal and motile spermatozoa with a single insemination. The low per cycle pregnancy rate observed in this trial most likely indicates that higher amounts of normal motile spermatozoa are needed to improve fertility. This study is part of the foundation of information that will be used to determine the dose response curve of fertility using cryopreserved equine semen. The use of cryopreserved spermatozoa from more stallions using this protocol and a wider array of spermatozoal dosages will help determine the magnitude of the variability between stallions in fertility.

The second experiment was performed to investigate factors that have been identified as contributing to poor conception rates using cryopreserved equine semen and falls under the broad objective.

Experiment 2: Quantitative and subjective ultrasonographic assessment of endometrial changes in mares inseminated with cryopreserved semen

Post breeding uterine inflammation:

Post breeding fluid accumulation is reported to be associated with subfertility in mares. Uterine fluid accumulation following breeding has been attributed to spermatozoal induced uterine inflammation and bacterial endometritis. Fluid accumulation is more common in mares with impaired uterine contractility which increases their susceptibility to bacterial infections and uterine inflammation. Subfertile mares are typically older mares that have poor uterine health as shown by fibrosis and inflammation in the endometrial tissues. The endometrium is the lining of the uterus. Undesirable uterine fluid accumulation in mares has been reported after natural service, and after AI with fresh, cooled or cryopreserved equine semen. Excess uterine fluid accumulation is believed to be more common after the use of cryopreserved semen.

For this trial the total number of spermatozoa was used to define the groups. The literature indicated that the most important determinant of sperm induced uterine inflammation is the total number of spermatozoa rather than the number of motile and normal sperm which is more a determinant of fertility.

Method: 63 estrous cycles in 50 mares were used to examine changes in uterine endometrial tissue characteristics of ultrasound images (echotexture) using computer assisted ultrasound image analysis before and after insemination with cryopreserved semen. Data were collected during a breeding trial at Bar C ranch

in Manitoba and from research mares housed at the WCVM. Mares that received an extender infusion were then bred with cryopreserved semen.

Mares were teased by a stallion and examined daily in estrus. Endometrial echotexture was evaluated at 3 time points: hCG administration, AI, and 12 hours post AI. A 5 mHz probe was used (Medison Sonovet 600) and the gain and power settings were kept constant for each examination.

The 3 groups are listed showing the total number of spermatozoa and the number of PMMN spermatozoa in the dose. The total number of spermatozoa were group 1: 1.2 billion (250 PMMN) n = 19, group 2: 750 million (100 PMMN) n = 19 and group 3: 200 million (25 PMMN) n = 12. which correspond to the groups 1 - 3 mentioned in the breeding trial respectively. In control cycles (n = 8) mares were infused with extender. Five mare cycles where insemination was not performed were used to determine endometrial changes in non-bred mares.

The number of straws varied per insemination dose. To standardize insemination volume, cryopreserved semen for each insemination was pooled and extended for a total volume of 20 ml using a Kenney type extender. In the 8 extender control cycles 10 mls of cryopreservation extender was mixed with 10 mls of Kenney type extender.

Subjective assessment: The examiner was blinded to treatment and the previous day's results. Notations of the amount (mm) of free uterine fluid, the overall degree of endometrial edema (graded on a scale of 0 to IV with 0 equal to none, and IV equal to intense edema) and the presence of hyperechoic flecks or speckles on the ultrasound examination of the uterus were noted.

Objective assessment: Cross sectional ultrasound images of the body midpoint and tip of the uterine horns were stored on S-VHS videotape, and imported into a computer assisted image analysis program (Synergyne, Roger Pierson, Royal University Hospital, Saskatoon, Sk, Canada). Each ultrasound image is composed of many small boxes called pixels. Four spots (each spot is a 20 mm circle) were placed on the area of the image that corresponded to the endometrium. The spots were placed in the four quadrants of the image. The combined area of these spots accounted for 20% of the endometrium in the image. The pixels within the four selected spots on the endometrium of the ultrasound image were assigned a number by the computer program which corresponding to the shade of grey of the pixel (the computer recognizes 256 shades). The pixels contained within the endometrial spots were averaged. An average of the pixels in the spots was then calculated for a mean pixel value. Mean pixel values were the data acquired from the endoemtrium of the images for the comparisons.

Statistics: The effect of time on subjective endometrial edema grade and the effects of time and uterine location (uterine body, ipsilateral or contralateral horn) on objective endometrial pixel values were analyzed with general linear models (GLM) for repeated measures, when significant differences were detected, post hoc analysis included paired T tests and Wilcoxon signed rank tests. The effects of insemination treatment on the presence of hyperechoic flecks and changes in edema grade and numerical pixel values from AI to post breeding examination were examined using Kruskal Wallis one way ANOVA. (A paper prepared for submission is listed as Appendix B).

Results:

Data:
Data from 5 cycles were lost because the images were not of sufficient quality for analysis or they were lost. Data from 58/63 cycles were used.

Subjective assessment: A general linear model for repeated measures was used to evaluate the change in subjective endometrial edema grade over time. This comparison was significant ($p<0.001$). Post hoc comparisons indicated a decrease ($p<0.004$) in edema from III at hCG administration to II at artificial insemination. There was no further change noted from AI to the post breeding examination. There was no difference between insemination treatments.

Slight fluid accumulation was observed only at the post breeding examination. Hypoechoic fluid accumulation of less than 10 mm was noted in the uterine body during 3 cycles:, one each in control extender, group 1,and group 3. The mare with fluid accumulation in the group 1 1.2 billion (250 million PMMN) group became pregnant. The presence of hyperechoic flecks was not associated with fluid accumulation.There was no effect of treatment on the presence of flecks or fluid accumulation.

Objective assessment: The GLM of mean numeric pixel values of the endometrium using time and uterine location showed a significant effect of time (p 0.007), but not uterine location. Post hoc analysis showed a significant difference in mean numeric pixel values between hCG and AI but not AI and post breeding examination. An increase in numerical pixel values was noted at each of the uterine locations between hCG and AI: uterine body $p < 0.001$, ipsilateral horn to the dominant follicle $P = 0.003$, contralateral horn to the dominant follicle $p= 0.03$.There were no differences between insemination treatments. In contrast to the subjective assessment of endometrial edema where an overall uterine score was given, in the objective assessment of endometrial pixel data 3 uterine locations were compared. No differences were found between uterine locations during the evaluation of mean pixel values regardless of the location of the dominant follicle.

Take home message:
A decrease in edema or an increase in pixel values is associated with impending ovulation during mare cycles where hCG is administered. The decrease in subjective endometrial edema gradecorresponds to a loss of endometrial edema. The increase in observed in mean numeric pixel values corresponds to a change in tissue echotexture. This is the first report to evaluate changes in endometrial edema and echotexture in mares treated with hCG. It is recommended that the pattern of change in the endometrial edema and mean numeric pixel values is observed and recorded when using hCG to induce ovulation in mares. If a decrease in edema is observed it indicates the mare is responding to the treatment and is likely to ovulate. This is important as the commercially available semen costs more than $1000 per dose, and insemination at the wrong time is unlikely to result in pregnancy.

The presence of a dominant follicle did not appear to alter the appearance of the endometrial echotexture of the uterine horn of the same (ipsilateral) side. This suggests that the dominant follicle does not exert a local effect on the endometrium.

Only a small amount of fluid was detected in young virgin mares following breeding with frozen semen. No differences were found between treatments, which suggested no dose dependent effect detected by ultrasonography, or computer assisted image analysis of the endometrium. Excess fluid accumulation has been reported by many practitioners following breeding with frozen semen in horses. Excess fluid accumulation is usually associated with subfertility and lower pregnancy rates. The data suggest that frozen semen, or frozen semen extender used alone is not very inflammatory in young virgin mares, whom have a healthy uterine status. Excess inflammation after insemination with frozen semen is likely an indication of subfertility.

General Discussion:

This report describes how equine frozen semen may be prepared and evaluated using the similar standards to bulls. This is important for the horse industry as many would suggest that equine cryopreserved semen should not be examined with the same quality control standards as the bull. It has been our experience that semen of acceptable qualtity will meet the bovine guidelines with the possible exception of the percent of normal sperm in the sample. In the bull where there is a greater economic incentive for fertility, greater than 70% of the spermatozoa are expected to have normal morphology (shape), but in stallions the average percentage of normal spermatozoa is 55% and should be used in place of 70% morphologically normal spermatozoa.

Conclusion: The dose response curve of insemination dose to per cycle pregnancy rate requires further investigation.

Report of results:
Data have been presented at the Red Deer Horse Owners and Breeders Conference, and have been submitted to the Society for Theriogenology for presentation at their international meeting. Preliminary results were published in the Equine Health Research Fund's newsletter called Horsehealthlines which was circulated with the Saskatchewan Horse Federation's Publication "Showtimes".

Future studies:

More data should be collected using more single insemination dose trials from more stallions to help further delineate the dose response curve for per cycle pregnancy rates.

The following pages contain a set of recommendations or guidelines that we have created for horse owners and breeders in Saskatchewan that wish to use cryopreserved equine semen. The information generated by this project should enable Saskatchewan horse breeders to use cryopreserved semen in a more knowledgeable fashion, and thereby making it more affordable and profitable.

Guidelines for the use of Cryopreserved Equine Semen

These guidelines are for a general population of mares

Semen Evaluation

The guidelines for the assessment of the quality of bovine cryopreserved semen using in vitro methods may be adapted for used with equine cryopreserved semen. The assessment of quality includes a determination of the progressive motility, the percentage of normal spermatozoa, the percentage of acrosome intact spermatozoa and the longevity of the spermatozoa when held at body temperature for 2 hours. The in vitro evaluation of the semen is intended to assess the potential fertility of the sample, but is not a guarantee of fertility. Characteristics of acceptable quality cryopreserved equine semen includes: greater than 55% normal spermatozoa, greater than 30% progressively motile spermatozoa at 10 minutes post thaw and greater than 20% progressively motile spermatozoa after 2 hours, and greater than 70% intact acrosomes at 2 hours post thaw. The percent normal spermatozoa is lower than that of the bull where 70% would be considered acceptable. The wide variability in the quality of commercial equine semen is problematic, therefore before purachasing equine cryopreserved semen an evaluation of the post thaw characteristics of this batch should be reviewed.

Synchronization of Estrus

Synchronization of estrus is an integral part of a successful AI program. Ovulation induction agents such as hCG (2000 IU IM) may be used to make the time of ovulation easier to predict in estrous mares. This drug is administered when a mare is showing behavioral estrus, and when she has mild moderate uterine endometrial edema, and a 35 mm follicle or larger. When hCG is used, insemination is usually performed about 36 hours after injection, as this is the mean time to ovulation. In cycles where hCG is used there is normally a decrease in endometrial edema from moderate edema (grade III) to mild edema (grade II) prior to ovulation. Ultrasound examination is used to determine the follicle size, and the degree of endometrial edema.

Timing of Insemination

Semen should be inseminated close to ovulation. The ideal window is likely to be 6 hours before to 6 hours after ovulation. Insemination greater than 12 hours before or 12 hours after insemination may result in decreased pregnancy rates. When hCG is used, insemination is usually performed about 36 hours after injection.

Preparation of Semen

Semen should be thawed according to the directions provided by the supplier. Cryopreserved semen should be diluted to about 20 ml in Kenney type extender to decrease the concentration of the semen. The extender used in the process of cryopreservation is not very inflammatory in the

mare's uterus. The dilution of the frozen semen is currently recommended to decrease the inflammatory response to the spermatozoa in the mare's uterus. The concentration and total number of spermatozoa are the most important determinants of uterine inflammation. We recommend dilution of the thawed cryopreserved semen in prewarmed Kenney's extender to make an infusion volume of about 20 cc.

Post Breeding Fluid Accumulation

Post breeding fluid accumulation following frozen semen has not been detected in young virgin mares with a healthy endometrial condition. Post breeding fluid accumulation is not a normal finding following insemination with frozen semen. Mares with a significant amount of fluid should receive treatment to remove the fluid, or the per cycle pregnancy rate may be decreased. Post breeding fluid accumulation is best monitored through the use of ultrasound.

Insemination dose

Per cycle pregnancy rates are generally lower substantially lower than with the use of fresh or chilled semen. The minimal number of normal and motile spermatozoa required is greater than 250 million per insemination, and likely is in excess of 750 million.

Appendix A
Preparation of Frozen Semen
General

Semen will be collected using a Missouri artificial vagina. The ejaculatory volume will be measured. Semen will be extended 1:1 with a Kenny type extender or HEPES buffer and layered over a small amount (0.4 ml) of egg yolk glycerol (SMEG), or EDTA lactose buffer in sterile test tubes. The extended semen will be centrifuged at 400 x g for 15 minutes and the supernatent removed. The pellet will be resuspended in SMEG buffer and a final dilution made. This mixture will be cooled to 4 C over one hour and then loaded into precooled prelabeled 0.5 ml straws at a concentration of 400 million sperm cells / ml. The straws will be suspended 5 cm over liquid nitrogen for 10 minutes and then plunged. Straws will be thawed at 37 C in a water bath for 40 sec. The Equine Frozen Semen evaluation of the WCVM will be performed on each ejaculate. Briefly semen is thawed at 37C and diluted into 10 mls Kenny's extender. Evaluations are performed at 0, 1, 2 hours. Motility and acrosome status are evaluated using phase contrast or DIC optics respectively. Morphology and live / dead status is assessed using eosin - nigrosin stain at 400x (oil immersion). Sperm cells must be a minimum of 25% motile with >70% intact acrosomes at 2 hours post thaw to be used in the project.

Straws will be emptied into a vial and motility evaluated. The thawed frozen semen will be diluted in Kenney's extender to a final volume of 10 ml. Semen will be diluted to provide an insemination dose of 250, 100, 25 million progressively motile, morphologically normal, acrosome intact (PMMN) sperm cell in 10 mls of extender.

Quantitative and subjective ultrasonographic assessment of endometrial changes in mares inseminated with cryopreserved semen

Authors

Theresa E. Burns DVM

Department of Herd Medicine and Theriogenology

Western College of Veterinary Medicine, University of Saskatchewan

52 Campus Drive

Saskatoon, Sk., Canada

S7N 5B4

(306) 966-7166

Roger Pierson MSc. PhD

Director, Reproductive Biology Research

Department Obstetrics, Gynecology and Reproductive Medicine, College of Medicine,

University of Saskatchewan

Saskatoon, Sk.

S7N 5B4

Claire C. Card DVM PhD

Department of Herd Medicine and Theriogenology

Western College of Veterinary Medicine, University of Saskatchewan

52 Campus Drive

Saskatoon, Sk., Canada

S7N 5B4

Take Home Message

Fluid accumulation or ultrasonographically visualized uterine changes do not occur in reproductively normal mares after a single periovulatory insemination of cryopreserved semen at doses up to 1.2 billion total spermatozoa.

Introduction

Physiologic transient postbreeding uterine inflammation is a natural process which helps the mare remove excess spermatozoa, seminal plasma, bacteria and debris introduced at breeding. This physiologic uterine inflammation peaks at 12 hours and abates by 24 – 36 hours postbreeding. Failure to clear inflammation is associated with lower pregnancy rates and is evident clinically by excess uterine fluid accumulation, and poor uterine tone. The use of cryopreserved semen has been associated with postbreeding fluid accumulation. It is hypothesized that the process of semen cryopreservation may increase the inflammatory nature of the inseminate through the removal of seminal plasma, alterations in sperm cell membranes, and intolerance to components of the freezing extenders. Increased numbers of neutrophils have been recovered from uterine flushings of mares inseminated with concentrated frozen-thawed semen.[1] Increased uterine inflammation is a possible etiology for poor fertility of equine cryopreserved semen.

The purpose of this study was to monitor ultrasonographically detectable endometrial changes surrounding ovulation induction and insemination with cryopreserved semen in young, reproductively healthy mares. Ultrasonographically detectable endometrial edema, fluid accumulation, and the uterine fluid characteristics

were monitored as practical, clinical indicators of uterine inflammation. Change in subjective endometrial edema grade associated with ovulation induction and insemination with cryopreserved semen was examined. Numerical pixel values of ultrasound images were obtained using a previously validated computer assisted image analysis[a] and were used to quantify changes in endometrial echotexture.

Materials and Methods

Fifty mares were used for a total of sixty-two estrous cycles to examine changes in uterine echotexture before and after insemination with cryopreserved semen. All mares were nulliparous, less than five years of age and had clinically normal reproductive tracts determined by rectal and ultrasonographic exam and evaluation of perineal conformation. Semen used for insemination was obtained from eleven ejaculates collected over an eight-week period from one stallion and processed using one cryopreservation protocol.

Mares were synchronized with two 5 mg subcutaneous injections of prostaglandin[b] administered fourteen days apart and randomized to one of four treatment groups: control (n=12), 200 million total spermatozoa (n=12), 750 million (n=19) or 1.2 billion (n=19) total spermatozoa. The control group was subdivided into extender infused (n=8) and non-inseminated (n=4) groups. All control mares were treated with 5mg of prostaglandin[b] administered subcutaneously at 7 days following treatment and re-randomized to the 750 or 1.2 billion spermatozoa insemination groups.

Transrectal ultrasonographic reproductive exams were performed daily on each mare from the onset of behavioral estrus until ovulation. Uterine and cervical tone,

follicle diameter, endometrial edema grade and the character of uterine fluid and hyperechoic flecks were noted. Estrous mares with a follicular diameter of at least 35 mm and endometrial edema grade two or greater, were injected with 2000 IU human chorionic gonadotropin (hCG)[c] intramuscularly. Artificial insemination was performed 32-36 hours after hCG treatment. Immediately prior to insemination the inseminate volume was standardized to 20 ml with a Kenney type extender.[d] Mares infused wth extender alone were treated with intrauterine infusion of ten milliliters of a skim milk egg yolk freezing extender combined with ten milliliters of Kenney extender.[d] Insemination to ovulation interval was recorded.

Endometrial echotexture was evaluated by subjective endometrial edema grading and objective computer-assisted image analysis at three times; hCG administration, artificial insemination and 14 hours post insemination. A subjective endometrial edema grade between 0 and 4 was assigned by assessment of the homogeneity of endometrial echotexture. Grade O corresponds to no edema while Grade 4 represents heavy intense edema. For computer assisted image analysis, digitally acquired cross sectional images of the uterine body and mid-region of the uterine horn ipsilateral and contralateral to the dominant follicle were recorded at the same time points on super VHS videotape and imported into the image analysis program. For each image, four 25 mm^2 spot measurements of numerical pixel values, comprising approximately 20% of the image, were made in the endometrial folds. Mean numerical pixel values per image were obtained by averaging the four spot measurements.

The effect of time on subjective endometrial edema grade, and the effects of time and uterine location (uterine body, ipsilateral or contralateral horn) on objective endometrial numerical pixel values were analyzed with general linear models for repeated measures. When significant differences in the overall model were detected, further comparisons were made using paired T tests for continuous data and Wilcoxin Sign Rank tests for ordinal data.

The effects of insemination treatment on presence of hyperechoic flecks, and changes in edema grade and numerical pixel values from artificial insemination to post breeding examination were examined using Kruskal Wallis one way ANOVAs.

Results

There was a significant decrease in subjective endometrial edema score from from grade 3 edema at hCG administration to grade 2 edema at artificial insemination ($p=0.002$). Subjective median endometrial edema grade did not change in the fourteen hour period from artificial insemination to post breeding examination.

There was a significant increase in objective endometrial numerical pixel values between hCG administration and artificial insemination at all uterine locations (uterine body $p<0.001$, ipsilateral horn $p=0.003$, contralateral horn $p=0.03$), but no change from insemination to post breeding examination. For each time point there were no differences in pixel densities in the uterine body and horns ipsilateral and contralateral to the dominant follicle.

There was no significant effect of insemination treatment on change in subjective edema grade or objective numerical pixel values in the uterine body, ipsilateral horn or contralateral horn from artificial insemination to post breeding examination.

Slight fluid accumulation was observed only at the post breeding examination time. Hypoechoic fluid accumulation of less than ten millimeters maximal depth was present in the uterine body of 5% (3/62) of mares: one mare in the extender infusion group and one mare in each of the 200 and 1.2 billion spermatozoa insemination groups. The mare with fluid accumulation in the 1.2 billion total spermatozoa insemination group became pregnant.

Hyperechoic flecks were observed only at the post breeding examination time. There was no effect of insemination dose on the occurrence of hyperechoic flecks. Hyperechoic flecks were not associated with fluid accumulation.

Eighty-one percent of mares ovulated in the interval from six hours before to twelve hours after insemination. Exclusion of mares that did not ovulate in this interval from statistical analysis did not alter the significance of any outcome.

Discussion

Mares in this trial had a significantly higher subjective edema grade at hCG administration (grade 3) than at artificial insemination (grade 2). These results indicate that mares induced to ovulate with hCG have declining endometrial edema as ovulation approaches similar to changes in natural cycles[2]. Computer assisted image analysis showed a significant increase in endometrial numerical pixel values associated with

declining edema grade. An increase in pixel score would be expected as tissue edema decreased prior to ovulation.

Post breeding fluid accumulation was monitored as an indicator of inflammation in this trial. Intrauterine fluid accumulation one to two days after ovulation is associated with significantly decreased pregnancy rates and mares susceptible to Streptococcus zooepidemicus challenge accumulate significantly more intrauterine fluid than resistant mares after ovulation.[4] Postbreeding uterine fluid accumulation was reported in 15% of naturally bred Thoroughbred mares in one field study, where fluid accumulation worsened with age.[5] Results of this present trial were interpreted to mean there is no significant fluid accumulation after breeding with cryopreserved semen at doses up to 1.2 billion total spermatozoa in young, healthy mares. Lack of fluid accumulation is likely associated with good endometrial and myometrial contractility along with cervical relaxation.[4,6]

The ultrasonographic appearance of uterine intraluminal hyperechoic flecks is characteristic of particulate matter or air bubbles. In this study, insemination with spermatozoa did not increase the incidence of hyperechoic flecks compared to insemination with extender alone, while non-inseminated mares did not develop flecks. Post breeding flecks may represent ultrasonographic reflections of microbubbles of air that entered the uterus at insemination or proteinaceous components of the extender. If flecks were a result leukocyte influx, the inflammatory response appeared to be within the physiologic range, as excess fluid accumulation did not occur.

Because significant intrauterine fluid accumulation did not occur in experimental mares, changes in uterine edema and numerical pixel values between insemination and

post breeding examination were analyzed to look for differences between treatment groups. However, there were no differences in change in edema grade or numerical pixel densities between groups. No quantitative ultrasonographically detectable changes in the endometrium were created by insemination with the doses of cryopreserved semen or freezing extender used in this trial.

High sperm cell concentration of cryopreserved semen has been hypothesized to increase uterine inflammation in the mare. Significantly fewer neutrophils were detected in uterine flushings after insemination with diluted cryopreserved semen than after insemination with concentrated cryopreserved semen.[1] In vitro, frozen spermatozoa and fresh spermatozoa at similar concentrations are equally chemotractant to neutrophils.[6] Dilution of cryopreserved semen with Kenney extender before insemination may be a contributing factor in minimizing fluid accumulation, inflammation, and ultrasongraphically endometrial changes in this trial, and may have practical application in the future. A standardized volume was used in this study because of the variable number of 0.5 ml straws used for the different insemination treatments

In conclusion, in mares induced to ovulate with hCG, edema decreases in the interval between hCG treatment and ovulation and this decrease can be quantified by computer assisted image analysis. Insemination with cryopreserved semen at doses up to 1.2 billion total spermatozoa, in a consistent volume of twenty milliliters, did not cause fluid accumulation in 95% of young, healthy mares. Differences in endometrial echotexture between extender or spermatozoal insemination at doses up to 1.2 billion total cryopreserved cells were not detectable by subjective or computer-assisted analysis. In clinical practice, marked ultrasonographic inflammation as determined by

fluid accumulation after insemination with cryopreserved semen may suggest underlying uterine pathology and impaired myometrial contractility in the mare or may result from management practices used in insemination of cryopreserved semen.

References

1. Kotilainen, T., Huhtinen, M., and Katila, T. Sperm-induced leukocytosis in the equine uterus. Theriogenology 1994; 41: 629-636.
2. Griffin, P.J. and Ginther, O.J. Research applications of ultrasonic imaging in reproductive biology. J Anim Sci 1992; 70: 953-972.
3. McKinnon, A.O., Squires, E.L., and Voss, J.L. Ultrasonic evaluation of the mares' reproductive tract-part II. Compend Cont Educ Pract Vet 1987; 9: 472-480.
4. LeBlanc, M.E., Neuwirth, L., Asbury, A.C., and et.al. Scintigraphic measurement of uterine clearance in normal mares and mares with recurrent endometritis. Equine Vet J 1994; 26: 109-113.
5. Zent,W., Troedsson, M.H., Xue, Jin-Liang. Postbreeding uterine fluid accumulation in a normal population of Thoroughbred mares: a field study. Proceedings. 44st Annu Conv Am Assoc Equine Practnr 1998; 64 - 65
6. Troedsson, M.H., Crabo, B.G., Naridin, N., and et.al. Mating-induced endometritis: Mechanisms, clinical importance and consequences, in Proceedings. 41st Annu Conv Am Assoc Equine Practnr 1995; 11-12.

Footnotes

a) Synergyne computer assisted image analysis system

R.Pierson, Department of Obstetrics, Gynecology and Reproductive Medicine, Royal University Hospital, Saskatoon, Sk. Canada S7N 5B4

b) The Upjohn Company-Animal Health Division

40 Centennial Road, Orangeville, On. Canada L9W 3T3

c) Ayerst Laboratories

400 Michener Road, Guelph, On. Canada N1K 1E4

d) EZE Mixin semen extender, Animal reproduction systems

14395 Ramona Avenue, Chino, CA 91710